Thrombosis and Haemostasis © Schattauer Verlagsgesellschaft mbH (Stuttgart) 68 [illegible]–59 (1992)

Comparison of Recombinant Tissue Factor Pathway Inhibitors Expressed in Human SK Hepatoma, Mouse C127, Baby Hamster Kidney, and Chinese Hamster Ovary Cells

Exhibit 2

Tze-Chein Wun[1]*, Kuniko K. Kretzmer[1], Mark O. Palmier[1], Kathleen C. Day[1], Margaret D. Huang[1], Dean J. Welsch[1], Charles Lewis[1], Richard A. Wolfe[1], James F. Zobel[1], Gary W. Lange[1], Ronald B. Frazier[1], Gary S. Bild[1], Michelle A. Peel[1], Robert E. Shell[1], Nancy A. Horn[1], Kurt D. Junger[1], Barbara A. Foy[1], Mark E. Gustafson[1], Richard M. Leimgruber[1], William F. Novotny[2], George J. Broze, Jr.[2], Yvonne E. Pyla[1], Paul J. Hippenmeyer[1], and Thomas G. Warren[1]

From the [1]Monsanto Corporate Research, Chesterfield, MO, the [2]Department of Hematology/Oncology, Washington University Medical School, St Louis, MO, USA

Summary

Recombinant tissue factor pathway inhibitor (rTFPI) has been expressed in four mammalian expression systems using human SK hepatoma, mouse C127, baby hamster kidney (BHK), and Chinese hamster ovary (CHO) cells as hosts. On sodium dodecyl sulfate polyacrylamide gel electrophoresis, the immunoaffinity purified rTFPIs all show broad bands and the mean molecular weight of SK hepatoma and C127 rTFPIs ($M_r \sim 38{,}000$) appear larger than those of BHK and CHO rTFPIs ($M_r \sim 35{,}000$). All these proteins inhibit factor Xa and appear to bind factor Xa with 1:1 stoichiometry. The ability of these proteins to inhibit tissue factor-induced coagulation in plasma was examined using a prothrombin time assay. The relative activities of SK rTFPI:C127 rTFPI:BHK rTFPI:CHO rTFPI were found to be 28:15:2.1:1. By Western blot using specific antisera against the amino- and carboxy-termini of TFPI as probes, it is found that all the immunoaffinity purified rTFPIs possess approximately equal amounts of the amino terminus, but the C127 and BHK rTFPIs are deficient in carboxy terminus and the CHO rTFPI is essentially devoid of this region of the protein. Mono S chromatography allowed separation of the full-length and the truncated molecules with high and low anticoagulant activities, respectively. The above results suggest that proteolysis of the carboxy terminus of TFPI occurs to different extent when TFPI is expressed in different cells and that the carboxy terminal region of the TFPI molecule is important for the inhibition of tissue factor-induced coagulation. Recombinant and non-recombinant SK hepatoma cells appear to produce TFPI with the least amount of proteolysis compared with other cell systems tested and SK TFPI functionally resembles TFPI circulating in the blood.

Introduction

Tissue factor (TF, tissue thromboplastin) is a membrane-bound glycoprotein that serves as the essential cofactor in the activation of the extrinsic pathway of coagulation (1). TF binds factor VII, and the resulting TF-factor VIIa complex converts factors X to Xa and IX to IXa eventually leading to the generation of thrombin and the formation of a fibrin clot.

Blood contains a protein inhibitor of TF-factor VIIa catalytic activity that requires the presence of factor X for its function (2, 3). This protein, named tissue factor pathway inhibitor (TFPI), [previously called extrinsic pathway inhibitor (EPI) (4), or lipoprotein-associated coagulation inhibitor (LACI) (5)], is a multivalent Kunitz type inhibitor (6). It has been postulated that in the presence of Ca^{2+} an inert TF:VIIa:Xa:inhibitor quaternary complex forms, resulting in the inhibition of the extrinsic coagulation pathway.

TFPI was initially isolated from the serum-free conditioned media of HepG2 cells, a human hepatoma cell line (7). Subsequently, TFPIs were also purified from two other liver-derived human cell lines, SK hepatoma cells and Chang liver cells (8). Using preincubated, end-point type assays such as a factor Xa inhibition assay and a three-stage TF inhibition assay, it was shown that the specific activity of all these proteins are equivalent. However, in a kinetic type of assay such as the prothrombin time (PT) assay, it was found that 1 unit of HepG2 TFPI gave a PT equivalent to those with 0.17 unit of SK TFPI or 0.71 unit of Chang TFPI (8). These data suggest that the isolated SK TFPI and Chang TFPI are kinetically 6- and 1.4-fold more potent than HepG2 TFPI. The reasons for the differences were not clear although post-translational modification was suggested to be the cause.

In an attempt to produce large amounts of TFPI protein for a variety of studies, we have cloned TFPI cDNA (6) and expressed the recombinant protein in several mammalian expression systems using human SK hepatoma, mouse C127, baby hamster kidney (BHK), and Chinese hamster ovary (CHO) cells as hosts. Here we report the expression and characterization of these rTFPIs.

Experimental Procedure

Materials

Ten-chamber cell factories were purchased from Nunc. Dulbecco's modified Eagles' medium and fetal bovine serum were from Gibco. Thromboplastin reagent (Simplastin® Excel) was obtained from Organon Teknika Corp. Bovine factor Xa, and Spectrozyme Xa were from American Diagnostica, Inc. SDS-PAGE 10–20% gradient gel was purchased from Integrated Separation Systems. Horse radish peroxidase (HRP) conjugated goat anti-rabbit Ig and HRP color development

Abbreviations: TFPI, tissue factor pathway inhibitor; MTX, methotrexate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; PT, prothrombin time.

* Correspondence to: Dr. Tze-Chein Wun, Monsanto Co. AA4E, 700 Chesterfield Parkway, Chesterfield, MO 63198, USA

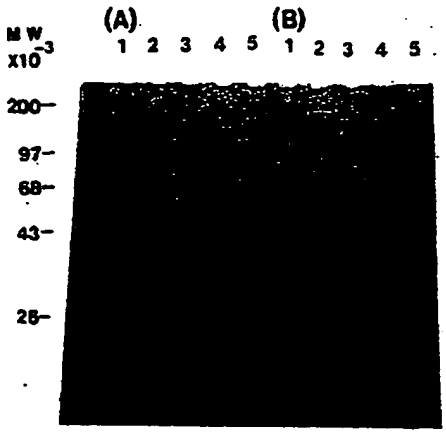

Fig. 1 SDS-PAGE of affinity purified rTFPIs. Electrophoresis was carried out on a 10–20% gradient gel. Each lane was loaded with 1.5 μg of protein. (A) lanes 1–5, samples were unreduced and not boiled; (B) lanes 1–5, samples were reduced in the sample buffer containing 2.5% 2-mercaptoethanol and boiled for 3 min. Lane 1, SK rTFPI; lane 2, C127 rTFPI purified by chromatography on a monoclonal anti-TFPI-Ig-Sepharose 4B column; lane 3, C127 rTFPI purified by chromatography on an anhydrotrypsin-Sepharose 4B column; lane 4, BHK rTFPI; lane 5, CHO rTFPI. The lane on the left is molecular weight markers

reagent (4-chloro-1-naphthol) were obtained from Bio Rad. Antiserum against TFPI N-terminus peptide was raised by immunizing rabbits with a synthetic peptide corresponding to amino acids 3–25 of the mature TFPI protein as described previously (8). Antiserum against TFPI C-terminal peptide was raised by immunizing rabbits with a synthetic peptide matching the C-terminal 12 amino acids of the mature TFPI protein.

Methods

Expression of rTFPI in Mouse C127 Cells

rTFPI was expressed in mouse C127 cells using a bovine papilloma virus vector as described before (9).

Expression of rTFPI in Chinese Hamster Ovary (CHO) and SK Hepatoma Cells

rTFPI was expressed in both CHO and SK hepatoma cells by methotrexate (MTX) selection of a dihydrofolate reductase amplification vector. The expression vector, pMON4594, is based on pSV2dhfr (10) and utilizes the SV40 Early promoter and the SV40 Late poly A addition site to control the expression of a 960 base pair TFPI cDNA fragment.

For expression of rTFPI in CHO DXB11 cells (11), cells were transfected with pMON4594 and dhfr-positive transfectants were selected by growth in nucleoside-free Dulbecco's Modified Eagle's Media (DMEM) supplemented with dialyzed fetal bovine serum. Individual colonies were picked and TFPI-producing transfectants were identified by immunoassay of the conditioned media. These colonies were then expanded and subjected to stepwise selection with increasing concentrations of MTX (12).

For expression of TFPI in SK hepatoma cells (13), cells were cotransfected with pMON4594 and pSV2neo and positive transfectants were selected by growth in DMEM supplemented with dialyzed fetal bovine serum containing 800 μg/ml of G418. Individual colonies were picked and TFPI-producing transfectants were identified by immunoassay of the conditioned media. These colonies were then expanded and subjected to stepwise selection with increasing concentrations of MTX.

Expression of rTFPI in Baby Hamster Kidney (BHK) Cells

For expression of rTFPI in BHK cells, the TFPI cDNA was ligated into pMON3360b which contains the herpes simplex virus IE175 promoter and the SV40 late polyadenylation signal such that expression of TFPI is under control of the IE175 promoter. This plasmid was transfected into a BHK cell line which was previously engineered to express the herpes simplex virus VP16 transactivator (P. Hippenmeyer et al., manuscript in preparation) along with plasmid pMON1118 which has the hygromycin phosphotransferase gene (14) under the control of the SV40 promoter. Cells were selected for resistance to hygromycin B, expanded and assessed for TFPI expression by immunoassay.

Isolation of rTFPIs from Cell Conditioned Media

The TFPI-producing cell lines were cultured in cell factories in DMEM containing 10% fetal bovine serum until confluence and were then switched to serum free DMEM-based media containing 50 units/ml aprotinin. The conditioned media were collected every 2–3 days and the TFPI-related proteins were purified from the concentrated conditioned media by immunoaffinity chromatography on a monoclonal anti-TFPI-Ig-Sepharose 4B column as described previously (9). A preparation of C127 rTFPI was also isolated by affinity chromatography on an anhydrotrypsin-Sepharose 4B column as follows. The serum-free conditioned medium was adjusted to 50 mM $(NH_4)_2SO_4$, filtered through a 0.2-μm filter and concentrated 30-fold using an Amicon YM30 radial cartridge. The concentrate was subjected to ammonium sulfate precipitation. Protein precipitated between 23–90% saturation of ammonium sulfate was collected and dialyzed against phosphate-buffered saline containing 20 mM Na_2SO_4. Triton X-100 was added to a final concentration of 0.05% and the solution was clarified by centrifugation at 40,000 × *g* for 1 h. The supernatant was chromatographed using an anhydrotrypsin-Sepharose 4B column [12 ml gel, prepared according to Ishii et al. (15)] equilibrated in phosphate-buffered saline containing 20 mM Na_2SO_4, 0.05% Triton X-100 (buffer A). The column was washed with 80 ml of buffer A and 80 ml of the same buffer without Triton X-100. The bound protein was eluted with 1.5 M NaSCN in three column volumes. The eluted protein was concentrated and dialyzed against a solution containing 0.15 M NaCl and 20 mM Na_2SO_4. The recovery of TFPI was approximately 60%.

Mono S Chromatography

The immunoaffinity purified TFPIs were dialyzed into S-buffer (20 mM Na-acetate, pH 4.5, 6 M urea). Two ml of samples were injected into a Mono S HR5/5 column using FPLC system (Pharmacia). Proteins were eluted with a 0–1 M NaCl gradient in S-buffer in 50 1-ml fractions at a flow rate of 1 ml/min.

Prothrombin Time (PT) Assay

PT assay was performed on a Fibrometer or a Coag-A-Mate timer. In the Fibrometer assay, 0.1 ml of plasma supplemented with purified TFPI

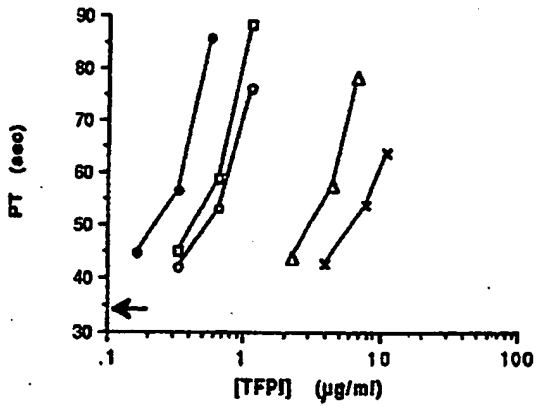

Fig. 2 Effect of affinity purified rTFPIs on the PTs of plasma. A normal plasma was supplemented with indicated concentrations of purified rTFPIs and the PT was measured using 1:240 dilution of TF on a Coag-A-Mate timer. The arrow indicates the PT of the plasma without exogenously added rTFPI. (●), SK rTFPI; (□), C127 rTFPI purified by chromatography on an anti-TFPI-Ig-Sepharose 4B column; (○), C127 rTFPI purified by chromatography on an anhydrotrypsin-Sepharose 4B column; (△), BHK rTFPI; (×), CHO rTFPI

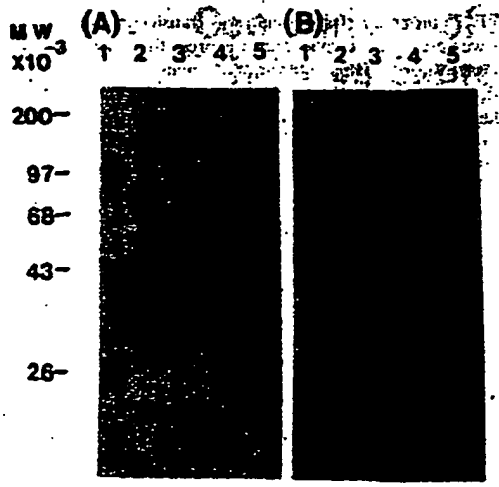

Fig. 3 Western blot of purified rTFPIs. SDS-PAGE was carried out on a 10–20% gradient gel. Each lane was loaded with 2 μg of purified proteins. Proteins were electrophoretically transferred onto nitrocellulose papers. The nitrocellulose papers were probed with antisera against the N-terminus (A), or the C-terminus (B) of TFPI, followed by HRP conjugated goat anti-rabbit Ig. Lane 1, SK rTFPII; lane 2, C127 rTFPI; lane 3, BHK rTFPI; lane 4 CHO rTFPI

or control buffer was incubated in the well at 37° C for 1 min and 0.2 ml of TF (diluted 1:60 or 1:120 into a solution containing 75 mM NaCl, 12.5 mM $CaCl_2$, and 0.5 mg/ml bovine serum albumin) was added to initiated the clotting. PT assay using the Coag-A-Mate timer was carried out according to the manufacturer's procedure employing 1:240 dilution of TF.

Amidolytic Assay of Factor Xa Inhibitory Activity

Inhibitory activity against bovine factor Xa of the purified rTFPIs was assayed by amidolysis of Spectrozyme Xa as described before (8).

Electrophoresis and Western Blotting

SDS-PAGE was performed using precasted 10–20% gradient gel (Integrated Separation system) at 100 volt. Proteins were transferred from the polyacrylamide gel onto nitrocellulose papers using a mini Trans-Blot cell (Bio Rad) according to the manufacturer's instruction. The nitrocellulose paper was sequentially soaked in 5% non-fat milk for 40 min, incubated with 1:200 dilution of antiserum for 1 h, and shaken in 1:1,000 dilution of HRP conjugated goat anti-rabbit Ig for 40 min, all at room temperature. Tris-buffered saline containing 0.05% Tween 20 was used for diluting the above reagents and for washing the nitrocellulose paper after each step. A solution was prepared by mixing 30 mg 4-chloro-1-naphthol, 10 ml of methanol, 30 μl of 30% H_2O_2, and 40 ml of Tris-buffered saline, and used for developing color on the nitrocellulose paper.

Protein Determination

The concentration of protein was determined by absorbance at 280 nm and by quantitative amino acid analysis after HCl/vapor phase hydrolysis at 110° C for 24 h.

Results

Expression of rTFPI in Mammalian Cell Hosts

As described previously (9), the highest TFPI producing C127 cell line transfected with a bovine papilloma virus vector secretes 2 μg/10^6 cells each 24 h. In this study, stepwise methotrexate selection of the dhfr negative CHO DXB11 cells up to 500 μM resulted in expression levels of up to 10 μg/10^6 cells each 24 h. Stepwise methotrexate selection of the dhfr positive SK hepatoma cells was only successful up to 0.8 μM MTX which produced expression levels of 2 μg/10^6 cells each 24 h. Transfection of a BHK cell line carrying the herpes simplex virus VP16 transactivator with a plasmid containing TFPI cDNA under the control of herpes simplex virus IE175 promoter resulted in an expression level of approximately 1 μg/10^6 cells each 24 h.

Isolation of rTFPIs and Comparison of their Properties

The rTFPI producing cell lines were cultured in serum containing media in cell factories. Upon confluence, the media were changed to serum-free and were replaced with fresh media every 2–3 days. All the cell lines survived under the serum-free condition for more than 1 month and maintained fairly stable secretion of TFPI into the media. The conditioned media all contain greater than 1 μg/ml of TFPI. To isolate rTFPIs, we processed and chromatographed the media on a monoclonal anti-TFPI-Ig-Sepharose 4B column as described in Methods. A preparation of C127 rTFPI was also isolated by affinity chromatography on an anhydrotrypsin-Sepharose 4B column. Fig. 1 shows the SDS-PAGE analysis of the isolated proteins under non-reducing (panel A), and reducing (panel B) conditions. Like the non-recombinant TFPIs reported previously (8), all the rTFPIs give diffuse bands due to microheterogeneity, and there appears to be two distinctive molecular weight classes of molecules. For convenience reason, the major species of rTFPIs isolated from SK hepatoma and C127 cells are referred to as M_r = 38,000 (lanes 1–3) and those from BHK and CHO cells as M_r = 35,000 (lanes 4 and 5). Under reducing condition, part of the M_r = 38,000 molecules purified from C127 cell by anhydrotrypsin-Sepharose 4B chromatography appear to degrade (see the smear under the major band in [B] lane 2), suggesting internal cleavage of part of the molecules.

Factor Xa Inhibitory Activity of Affinity Purified rTFPIs

Purified rTFPIs were incubated with bovine factor Xa and the residual uninhibited factor Xa was quantitated with a chromogenic substrate, Spectrozyme Xa. The stoichiometry (TFPI:Xa) of inhibition were found to be 1.2:1, 1.1:1, 1.1:1, and 0.98:1 for SK hepatoma-, C127-, BHK-, and CHO-derived TFPI, respectively.

Inhibition of TF-Induced Coagulation by Affinity Purified rTFPIs

Addition of TF to plasma in the presence of Ca^{2+} induced a time-dependent coagulation of plasma which forms the basis of the prothrombin time (PT) assay. By keeping the TF concentration constant, PT assay can be used to assess the extent and the rate of inhibition of TF by TFPIs (8). Fig. 2 shows the PT as a function of the concentrations of rTFPIs added to plasma. The relative potencies of these purified proteins in the inhibition of coagulation can be estimated from the concentrations of rTFPIs that give the same PT. For example, the relative potencies of SK:C127 (anti-TFPI-Ig purified):C127 (anhydrotrypsin purified): BHK:CHO are 28:15:12:2.1:1 at PT = 60 s. Variability of specific activity was observed in different preparations of C127-, BHK-, and CHO-TFPIs which may be due to different extent of proteolysis of the TFPI molecules during cell culture and purification (see below).

Western Blot Analysis of Purified rTFPI

The appearance of diffuse protein bands and the differences in the apparent molecular weights of the purified rTFPI in SDS-

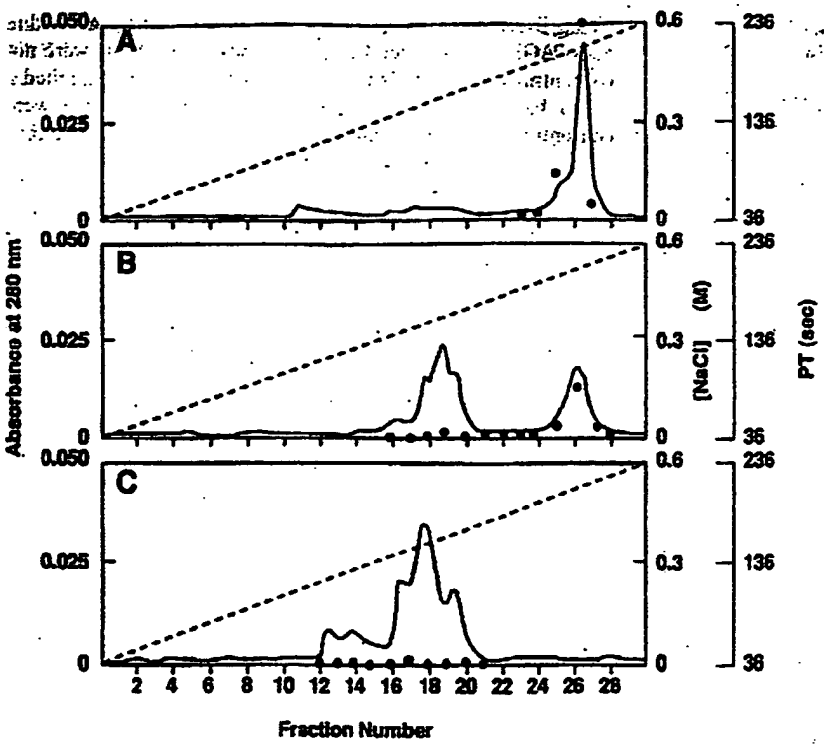

Fig. 4 Mono S chromatography of the immunoaffinity isolated SK-, C127, and CHO-rTFPIs. Mono S chromatographies were carried out using a 0–1 M NaCl gradient in a buffer containing 20 mM Na-acetate, pH 4.5, 6 M urea. (——), absorbance at 280 nm; (——), NaCl gradient; (●), PT. PTs were measured by supplementing 100 µl of a normal plasma with 1 µl of indicated fractions and measuring the clotting time in a Fibrometer after addition of 200 µl of 1:120 dilution of TF

PAGE suggest variations in glycosylation and/or differential proteolysis. To investigate whether proteolysis produced the molecules with lower M_r, Western blotting was performed using antibody probes for the amino- and carboxyl-termini of TFPI. Fig. 3(A) shows that equal amounts of all purified rTFPIs give bands of similar intensity when probed with antiserum against a synthetic peptide corresponding to the amino-terminus of TFPI. In contrast, when the same amounts of the proteins were probed with antiserum against a synthetic peptide corresponding to the carboxy-terminus of TFPI [Fig. 3(B)], the intensity of the bands is strongest for SK rTFPI, weaker for C127 rTFPI, barely stained for BHK rTFPI, and no visible bands for CHO rTFPI. These results indicate that the C127-, BHK-, and CHO-rTFPIs are partially or completely missing the carboxy-terminal peptide possibly due to proteolysis. The fact that the relative activities of these rTFPIs in the PT assay match the extent of preservation of the carboxy-terminus suggest that the carboxy-terminus of TFPI is important for the inhibition of TF-induced coagulation by TFPI.

Fractionation of Full Length and Truncated TFPIs by Cation Exchange Chromatography

The carboxy-terminus of TFPI contains a stretch of positively charged amino acids (6), and therefore, truncation at the carboxy-terminus may significantly alter the ionic property of the molecule. To explore this possibility, we attempted to separate the full length and truncated forms of rTFPI by cation exchange chromatography. Fig. 4 shows the chromatograms for immunoaffinity isolated SK rTFPI (A), C127 rTFPI (B), and CHO rTFPI (C). The SK rTFPI elutes as a single major peak at approximately 0.55 M NaCl; the C127 rTFPI elutes at two positions, 0.4 and 0.55 M NaCl; and the CHO rTFPI elutes at around 0.4 M NaCl. PT assay shows that the activity is mainly associated with the proteins which elute at around 0.55 M NaCl and little activity is seen with the proteins that elute at 0.4 M NaCl (Fig. 4). Western blot analysis of the C127 rTFPI fractions from the Mono S chromatography (Fig. 5), shows that proteins which elute at 0.4 M contains amino-terminus of TFPI (panel [A], lanes 2–4), but is missing carboxy-terminus (panel [B], lanes 2–4). The proteins that elutes at 0.55 M NaCl are reactive toward antisera against both amino- and carboxy-termini of TFPI and they therefore contain full length TFPI (panels [A] and [B], lanes 5–6). Note that the proteins eluting at 0.4 M NaCl are lower in molecular weight than those elute at 0.55 M NaCl, consistent with the notion that the former is truncated at the carboxy-terminus. Further proofs that the lower molecular weight rTFPI was truncated at the carboxy-terminus were obtained by amino acid composition analysis and tryptic peptide mapping. The amino acid composition of the lower molecular weight rTFPI was deficient in lysine and arginine which existed in large number in the carboxy-terminus, and the tryptic peptide map of the lower molecular weight rTFPI lacked a carboxy-terminus peptide which existed in the full length rTFPI (data not shown).

The activities of the SK- and C127-rTFPIs which elute at 0.55 M NaCl were compared using the PT assay. Fig. 6 shows the effects of these proteins in the prolongation of PT. The result indicates that the two proteins possess rather similar PT activity. The specific activity of C127 protein is 87% that of SK protein as estimated by comparing the concentrations of proteins giving the same PT. This small difference may reflect a variation in the molecules (e.g. glycosylation) but is likely within the bounds of experimental error.

Comparison of Recombinant (r) and Non-Recombinant (nr) SK TFPI

In a previous study (8), we have shown that the M_r = 38,000 TFPIs isolated from non-recombinant SK hepatoma (nrSK), Chang liver, and HepG2 hepatoma cells possess different specific activity in the PT assay. The activity of nrSK TFPI was 6–7 fold that of Hep-G2 TFPI, and 5-fold that of Chang liver TFPI. Furthermore, the nrSK TFPI was found to be as active as

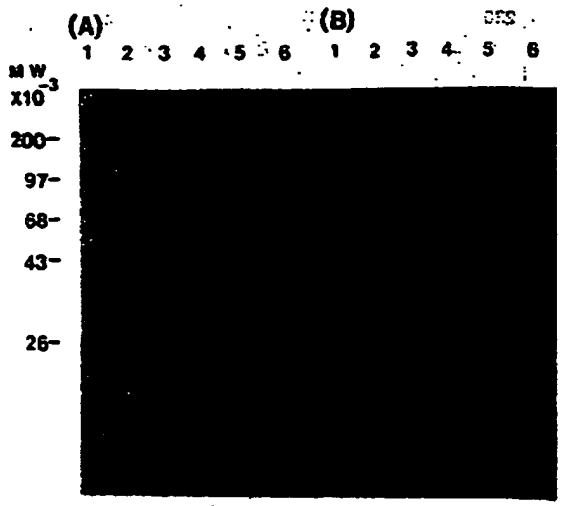

Fig. 5 Western blot of C127 rTFPI fractionated by Mono S chromatography. Mono S chromatography was carried out as described in Fig. 4 (B). Fifteen µl samples from fractions 27–29 and 35–36 were electrophoresed on a 10–20% gradient gel and electrophoretically transferred onto nitrocellulose papers. The nitrocellulose papers were probed with antisera against the N-terminus (A), or C-terminus (B), followed by HRP conjugated goat anti-rabbit Ig. Lane 1, molecular weight markers; lane 2, fraction 27; lane 3, fraction 28; lane 4, fraction 29; lane 5, fraction 35; lane 6, fraction 36

the TFPI present in human plasma or the immunoaffinity isolated plasma TFPI. These results suggest that the SK hepatoma cell is unique and it provides a source of TFPI that is closest or identical to the physiological TFPI. In this study, we have transfected the SK hepatoma cell to express a larger amount of TFPI (10–20 fold that of non-recombinant SK hepatoma cell), and isolated rSK TFPI. The rSK TFPI and the nrSK TFPI are compared using the PT assay. Fig. 7 shows that both TFPIs possess identical activity.

Discussion

In a previous study, TFPIs were purified from the culture media of non-recombinant HepG2 hepatoma, SK hepatoma, and Chang liver cells (8). The purified TFPIs showed a broad band in SDS-PAGE with a mean $M_r \sim 38{,}000$ when the media were not concentrated before immunoaffinity purification, but exhibited a broad band with lower $M_r \sim 35{,}000$ when the media were concentrated before chromatography. As much as 7-fold difference in specific activity was observed in the PT assay when $M_r \sim 38{,}000$ species of TFPIs from the above cells were compared. In view of the present finding, this suggests that SDS-PAGE may have failed to completely separate the $M_r \sim 38{,}000$ and 35,000 molecules due to the broadness of the bands and that the 7-fold difference in activity may have been due to differences in the integrity of the purified proteins. In this study, we have attempted to express recombinant TFPI proteins using four different mammalian host cells with the aims of achieving high level expression and obtaining a system that produces high specific activity TFPI molecules. The expression level was highest in the CHO/dhfr system which produced approximately 10 µg/10^6 cells each 24 h. The SK hepatoma/dhfr, C127/BPV, and BHK/VP16/IE175 systems were less efficient, producing 1–2 µg/10^6 cells each 24 h. The specific activities of the isolated recombinant TFPIs were again very different, with the SK TFPI ($M_r \sim 38{,}000$) having a specific activity 28-fold higher than that of CHO TFPI ($M_r \sim 35{,}000$). The C127- and BHK-TFPIs had intermediate activities and contained both classes of molecules. It appeared that the large difference in specific activity of these TFPIs is related to the extent of proteolysis at the carboxy-terminus of the molecules. Mono S seemed to be excellent in separating the full-length and the truncated form of TFPI.

Several post-translational modification occur in mammalian TFPIs, including glycosylation (3), sulfation of N-linked carbohydrate (3), phosphorylation at serine-2 (16), and limited proteolysis (8). The relative influence of these modifications on the specific activity of TFPIs remains to be rigorously established. However, several lines of evidence suggest that limited proteolysis of the carboxy-terminus has a great impact on the specific activity of TFPI. First, conversion from $M_r \sim 38{,}000$ to 35,000 occurred in some preparations of isolated TFPI after prolonged storage, accompanied by a large loss of activity; second, the isolated $M_r \sim 38{,}000$ and 35,000 TFPIs represent molecules with and without carboxy-terminus, respectively, and this difference is associated with a large variation of activity; and third, a carboxy-terminal mutant TFPI expressed in C127 system, which was

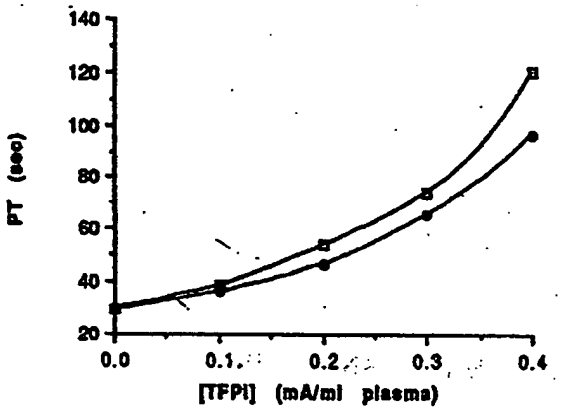

Fig. 6 Effect of full length SK- and C127-rTFPIs on the PT of plasma. Full length TFPIs were isolated by immunoaffinity purification followed by Mono S chromatography. Normal plasma was supplemented with indicated concentrations of full length SK- and C127-rTFPIs and the PTs were measured using 1:60 dilution of TF on a Fibrometer. One mA is defined as 1×10^{-3} absorbance unit of protein which is equivalent to 1.3 µg/ml of rTFPI as assayed by amino acid analysis. (□), SK rTFPI; (●), C127 rTFPI

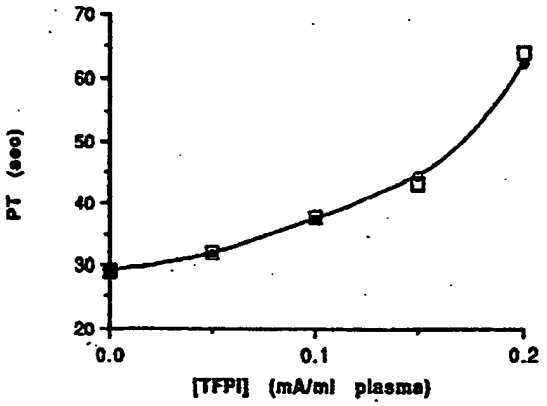

Fig. 7 Effect of rSK- and nrSK-TFPIs on the PT of plasma. Full length rSK- and nrSK-TFPIs were isolated by immunoaffinity purification followed by Mono S chromatography. Normal plasma was supplemented with indicated concentrations of rSK- and nrSK-TFPIs and the PTs were measured using 1:60 dilution of TF on a Fibrometer. The concentrations of the proteins were estimated by absorbance at 280 nm. One mA is defined as 1×10^{-3} absorbance unit of protein which is equivalent to 1.3 µg/ml of rTFPI. (●), rSK TFPI; (□), nrSK TFPI

truncated after leucine-252 and thus lacked the basic sequence KTKRKKQRVK (residues 254–265), possessed much lower activity than the full length C127 TFPI (17). It is not known at this time when the carboxy terminal proteolysis occurs. The fact that some purified preparations undergo degradation suggest that proteolysis may occur in the medium and during purification due to the presence of proteases. It remains to be established whether proteolysis also occurs inside the cells and whether addition of proteases inhibitors (other than aprotinin used in this study) in the cultures could prevent proteolysis of the carboxy-terminus. At this time, it is not clear whether differences in glycosylation in these molecules also influence their activity. However, we have obtained an unglycosylated TFPI and found that it possessed a specific activity similar or higher than the full-length SK TFPI (unpublished results), suggesting that glycosylation was not required for the TFPI activity.

It is apparent from the present data that the major site of proteolysis occurs in the highly positively charged carboxy-terminus and the loss of this segment of the molecule correlates with the decrease of the ability of TFPI to inhibit TF-induced coagulation. Carboxyl terminal truncation does not abolish the ability of TFPI to inhibit factor Xa. This is consistent with the finding that the second Kunitz domain is responsible for the binding of factor Xa (18). It is likely that the residual activity exhibited by the truncated TFPI in the PT assay is at least partly due to its anti-factor Xa activity since TFPI at high concentration also inhibits intrinsic coagulation as assessed by the activated partial thromboplastin time assay (19). These considerations taken together emphasizes the importance of TFPI's carboxy terminal domain in the inhibition of tissue factor-induced coagulation. Interestingly, a synthetic peptide corresponding to TFPI's carboxy terminal sequence possesses inhibitory activity against TF-induced coagulation (T.-C. Wun and Y. Konishi, unpublished result).

It has been shown that TFPI isolated from non-recombinant SK hepatoma cell possesses similar specific activity to the isolated human plasma TFPI (8). The calculated TFPI concentration of a normal pooled plasma based on the PT assay was equivalent to about 113 ng of SK TFPI/ml of plasma which closely matches the value (60–180 ng/ml, mean 113 ng/ml) obtained by other immunological and functional assay (3, 20, 21). Therefore, SK hepatoma TFPI appears to resemble functionally the TFPI circulating in plasma. In this study, we have further documented the functional similarity between the non-recombinant and recombinant SK hepatoma TFPIs (Fig. 7). In view of the difficulty in obtaining large quantities of purified plasma TFPI, recombinant SK hepatoma TFPI is potentially an attractive material for use as a reference standard.

During journal review of this paper, two other publications appeared which demonstrated that TFPIs derived from various sources are susceptible to proteolysis at the carboxy terminus resulting in large decreases of anticoagulant activity (22, 23). It is apparent from these and our studies that the purified products may show different extents of proteolysis depending on the process and scale of purification, aside from the cell line-dependence.

REFERENCES

1. Nemerson Y. Tissue factor and hemostasis. 1988; 71: 1–8.
2. Rapaport SI. Inhibition of factor VIIa/tissue factor-induced blood coagulation: with particular emphasis upon a factor Xa-dependent inhibitory mechanism. Blood 1989; 73: 359–65.
3. Broze GJ Jr. Girard TG, Novotny WF. Regulation of coagulation by a multivalent Kunitz-type inhibitor. Biochemistry 1990; 29: 7539–46.
4. Rao LVM, Rapaport SI. Studies on the mechanism of inactivation of the extrinsic pathway of coagulation. Blood 1987; 69: 645–51.
5. Broze GJ Jr, Warren LA, Novotny WF, Higuchi DA, Girard JJ, Miletich JP. The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action. Blood 1988; 71: 335–43.
6. Wun T-C, Kretzmer KK, Girard TJ, Miletich JP, Broze GJ Jr. Cloning and characterization of a cDNA coding for the lipoprotein-associated coagulation inhibitor shows that it consists of three tandem Kunitz-type inhibitory domains. J Biol Chem 1988; 263: 6001–4.
7. Broze GJ Jr, Miletich JP. Isolation of the tissue factor inhibitor produced by HepG2 hepatoma. Proc Natl Acad Sci USA 1987; 84: 1886–90.
8. Wun T-C, Huang MD, Kretzmer KK, Palmier MO, Day KC, Bulock JW, Fok KF, Broze GJ Jr. Immunoaffinity purification and characterization of lipoprotein-associated coagulation inhibitors from Hep G2 hepatoma, Chang liver, and SK hepatoma cells. J Biol Chem 1990; 265: 16096–101.
9. Day KC, Hoffman LC, Palmier MO, Kretzmer KK, Huang MD, Pyla EY, Spokas E, Broze GJ Jr, Warren TG, Wun T-C. Recombinant lipoprotein-associated coagulation inhibitor inhibits tissue thromboplastin-induced intravascular coagulation in the rabbit. Blood 1990; 76: 1538–45.
10. Subramani S, Mulligan R, Berg P. Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. Mol Cell Biol 1981; 1: 854–64.
11. Urlaub G, Chasin LA. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc Natl Acad Sci USA 1980; 77: 4216–20.
12. Schimke RT. Gene amplification in cultured animal cells. Cell 1984; 37: 705–13.
13. Fogh J, Trempe G. New human tumor cell lines. In: Human Tumor Cells In Vitro. Fogh J (ed). Plenum Press, New York 1975; pp 115–59.
14. Blochlinger K, Diggelmann H. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. Mol Cell Biol 1984; 4: 2929–31.
15. Ishii S-H, Yokosawa H, Kumazaki T, Nakamura I. Immobilized anhydrotrypsin as a specific affinity adsorbent for tryptic peptides. Methods Enzymol 1983; 91: 378–83.
16. Girard TJ, McCourt D, Novotny WF, MacPhail LA, Likert KM, Broze GJ Jr. Endogenous phosphorylation of the lipoprotein-associated coagulation inhibitor at serine-2. Biochem J 1990; 270: 621–5.
17. Wesselschmidt R, Likert K, Girard T, Wun T-C, Broze GJ Jr. Lipoprotein-associated coagulation inhibitor: The carboxy-terminus is required for optimal inhibition of factor Xa. (Submitted).
18. Girard TJ, Warren LA, Novotony WF, Likert KM, Brown SG, Miletich JP, Broze GJ Jr. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. Nature 1989; 338: 518–20.
19. Welsch DJ, Novotny WF, Wun T-C. Effect of lipoprotein-associated coagulation inhibitor (LACI) on thromboplastin-induced coagulation of normal and hemophiliac plasmas. Thromb Res 1991; 64: 213–22.
20. Sandset PM, Sirnes PA, Abildgaard U. Factor VII and extrinsic pathway inhibitor in acute coronary disease. Br J Haematol 1989; 72: 391–6.
21. Warr TA, Wam-Cramer BJ, Rao LVM, Rapaport SI. Human plasma extrinsic pathway inhibitor (EPI) activity: I. Standardization of assay and evaluation of physiological variables. Blood 1989; 74: 201–6.
22. Nordfang O, Bjorn SE, Valentin S, Nielsen LS, Wildgoose P, Beck T, Hedner U. The C-terminus of tissue factor pathway inhibitor is essential to its anticoagulant activity. Biochemistry 1991; 30: 10371–6.
23. Colburn P, Crabb JW, Buonassisi V. Enhanced inhibition of tissue factor by the extended form of an endothelial cell glycoprotein (an extrinsic pathway inhibitor). J Cell Physiol 1991; 148: 320–6.

Received November 4, 1991 Accepted after revision February 17, 1992